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Nanoleakage Inhibition Within Hybrid Layer Using New Protective Chemicals and Their Effect on Adhesion

ABSTRACT

Hybrid-layer degradation occurs because of acidic properties of currently used adhesive systems. Titanium tetrafluoride couples with tooth surface, and titanium compounds are not substituted. Caffeic acid phenethyl esther inhibits endogenous matrix metalloproteinases that cause hybrid-layer degradation. It was hypothesized that titanium tetrafluoride and caffeic acid phenethyl esther application on exposed dentine surfaces before adhesive applications would inhibit nanoleakage and hybrid-layer degradation without compromising the bond strength of the adhesives. In ultracut thin sections, human dentine–chemical agent–adhesive composite interfaces were observed under transmission electron microscope with complementary scanning electron microscopy. Microtensile bond strength tests were also accomplished. Titanium tetrafluoride and titanium tetrafluoride + caffeic acid phenethyl esther applications decreased bond strength values. Caffeic acid phenethyl esther showed decreased silver nitrate penetration for cements based on Bisphenol glycydilmethacrylate and methyl methacrylate, whereas cement based on 4-methacryloyloxyethyl trimellitate anhydride methyl methacrylate showed almost no infiltration. Caffeic acid phenethyl esther application before cementation could inhibit nanoleakage and biodegradation of the hybrid layer.

KEY WORDS: titanium tetrafluoride, caffeic acid phenethyl esther, hybrid layer, microtensile bond strength, nanoleakage.

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Nanoleakage throughout the hybrid layer and/or adhesive resin can be described as penetration of any substance into 20- to 100-nm-sized spaces present in the adhesive and/or tooth substrate (Nakabayashi, 1992; Nakabayashi and Takarada, 1992; Pashley *et al*., 1993). Although these spaces are too small to allow for bacterial penetration, they are large enough for enzymes to enter. Matrix metalloproteinases (MMPs) are a family of zinc-dependent endoproteinases whose enzymatic activity is directed against components of the extracellular matrix and so cause biodegradation of dentin (Jin *et al*., 2005; Magalhães *et al*., 2009). Transmission electron microscopy (TEM) images obtained with silver nitrate tracer demonstrated that water can pass from dentin, around resin tags, to form water-filled channels ("water trees") that project from the hybrid layer into the overlying adhesive (Tay *et al*., 2003; Hashimoto *et al*., 2004) that might act as potential sites for hydrolytic degradation of resin-dentin bonds (Sano *et al*., 1995). All marketed products have so far permitted some amount of nanoleakage and water-tree formation. Ideally, nanoleakage at the resin-dentin interface should be minimized or diminished. Penetration and polymerization of adhesive are essential to avoid biodegradation of resin-bonded dentin. Additionally, the adverse effects of esterase and MMPs at the resin-dentin interface should be blocked (Sano, 2006).

Titanium is a nontoxic element, and its pure metallic form is acknowledged as being biologically acceptable (Schlueter *et al*., 2007). Also, no side effects have been reported with the compound form titanium tetrafluoride (TiF_4). When hydrolysis of TiF_4 results at low pH, titanium has a strong tendency to couple with an oxygen atom of a phosphate group on the tooth surface (Tveit *et al*., 1983). Titanium compounds formed are so tightly bound that they are not easily substituted (Büyükyılmaz *et al*., 1997). TiF_4 might prevent the effects of enzymatic activity in dentin, and this may theoretically minimize the demineralization of dentin.

Caffeic acid phenethyl ester (CAPE) is a biologically active ingredient of propolis, with antioxidant, anti-inflammatory, antiviral, immunostimulatory, anti-angiogenic, anti-invasive, anti-metastatic, and carcinostatic activities. CAPE inhibits enzymatic activity of MMP-2 and MMP-9 (Jin *et al*., 2005).

Inhibiting demineralization can prevent osmotic process and nanoleakage and thereby stabilize long-term resin-dentin bond. It was hypothesized that conditioning dental substrates with TiF_4 and CAPE would inhibit nanoleakage at the hybrid layer. The objective of this study was to develop a pretreatment method for the exposed dentin, especially after tooth preparation, to form a resistant layer against demineralization by using different chemical agents.

MATERIALS & METHODS

Teeth Collection and Preparation

Intact fresh human third molars (N = 84) were collected with the informed consent of the donors, following a protocol approved by the ethical committee of Ege University, İzmir, Turkey. The teeth were stored in 0.1% thymol-saturated isotonic saline at 4°C to inhibit microbial growth, and they were used within 2 months following extraction. They were then randomly divided into 3 groups. Three luting resin materials based on Bisphenol glycydil-methacrylate (Bis-GMA; Variolink II, Ivoclar, Liechtenstein), 4-methacryloyloxyethyl trimellitate anhydride methyl methacrylate (4-META/MMA; Super-Bond C&B, Sun Medical, Japan), and methyl methacrylate (MMA; Multilink Automix, Ivoclar) as the main compositions were adhered onto the prepared dentin (n = 28 per group). Each luting resin cement group was further randomly divided into 4 subgroups of surface treatments (n = 7): TiF_4, CAPE, TiF_4 + CAPE, and control (*i.e.*, nonconditioned). Each tooth was sectioned perpendicular to its longitudinal axis under water cooling with a low-speed diamond saw (Isomet, Buehler, IL) to expose a flat midcoronal dentin surface. Each surface was ground finished with 600-grit silicon carbide abrasive paper under running water for 30 seconds before bonding (Tay *et al*., 2003).

Preparation and Application of the Chemical Agents

CAPE was synthesized by esterification of caffeic acid with phenethyl alcohol in the presence of p-toluenesulfonic acid (Jin *et al*., 2005) at the Pharmaceutical Research and Development Center of Ege University. CAPE solution with a concentration of 5% (wt/v) was prepared (pH, 6.5). TiF_4 was dissolved in deionized distilled water to achieve a concentration of 2.5% (wt/v; pH, 1.4). The proper concentrations for TiF_4 and CAPE were determined in a pilot study (Çömlekoğlu *et al*., 2009). CAPE and TiF_4 were applied for 60 seconds, each following acid etching in 4-META/MMA- and Bis-GMA-based cement groups and following acidic monomer application for MMA cement. For the TiF_4 + CAPE subgroups of all cement groups, TiF_4 was first applied, then CAPE.

Bonding Procedures

Ceramic specimens (8 × 8 × 6 mm, IPS E.max, Ivoclar) were etched with 4.9% hydrofluoric acid for 20 seconds (IPS Ceramic Etching Gel, Ivoclar), silanated for 60 seconds (Monobond-S, Ivoclar), and adhered onto the prepared dentin surfaces with the 3 adhesive cements under a constant load of 300 g. In 4-META/MMA cement groups, the monomer was activated by Catalyst S (Super-Bond C&B, Sun Medical) while the prepared dentin surfaces were etched with citric acid + $FeCl_3$ gel (Green Activator, Sun Medical) for 10 seconds. Then, polymer (L-Type Opaque, Sun Medical) was mixed with activated monomer and applied onto the dentin surfaces. For Bis-GMA cement groups, primer (Syntac Primer, Ivoclar) and adhesive (Syntac Adhesive, Ivoclar) were applied for 15 and 10 seconds, respectively, and gently air-dried. Subsequently, the bonding agent (Heliobond, Ivoclar) was applied, as followed by the application of the dual-polymerized resin cement according to the manufacturer's instructions. It was photopolymerized for 40 seconds from each aspect. For MMA cement groups, Primer A and B liquids were mixed in a 1:1 ratio, applied on prepared dentin surfaces with a microbrush for 15 seconds, and the luting material was directly syringed onto the ceramic and dentin surfaces. The margins were photopolymerized for 20 seconds and covered with oxygen-inhibiting gel (Liquid Strip, Ivoclar). The specimens were then thermocycled (×1500, 5°C to 55°C, 20 seconds).

Microtensile Bond Strength Test

Each tooth was sectioned with a slow-speed saw under water cooling into multiple beams (1 × 1 mm), with the nontrimming version for the microtensile bond strength (μTBS) test (Shono *et al*., 1999). After the very external cuts were eliminated, 4 beams were chosen from mesial, distal, buccal, and lingual sites of each tooth, yielding a total of 336 beams for all groups. These beams represented the areas of crown margins. Additional two beams from mesial and distal sides of each tooth were used for nanoleakage evaluation. The cross-sectional area at the site of failure was measured to the nearest 0.01 mm (Model CD-6BS, Mitutoyo, Tokyo, Japan). The beams were fixed to the alignment jig for parallel fixation in the universal testing machine (Shimadzu Autograph AGS-J 5kN, Kyoto, Japan) with cyanoacrylate glue (Model Repair II Pink, Dentsply-Sankin, Ohtawara, Japan) and stressed at a crosshead speed of 1 mm per minute until failure. Failure modes were evaluated at ×40 magnification with a stereoscopic microscope (Leica M205A, Leica Microsystems, Wetzlar, Germany).

Statistical Analysis

Two-way analysis of variance and Bonferroni correction (95% confidence interval) were used to determine statistical differences among μTBS values (SPSS 15.0). Chi-square test was used for failure analysis.

Nanoleakage Evaluation

Two additional separate beams were obtained from mesial and distal sides of the teeth in each cement subgroup. After rehydration in distilled water for 10 minutes, the beams were immersed in a tracer solution consisting of 50% (wt/v) ammoniac silver nitrate (pH, 9.5) for 24 hours (Tay *et al*., 2003). The silver-impregnated beams were rinsed with distilled water and placed in photodeveloping solution for 8 hours under a fluorescent light to reduce the diamine silver ion complexes into metallic silver grains (Kodak Professional D-76 developer, Kodak, Rochester, NY) within voids along the interface. The beams were then dehydrated in an ascending ethanol series (30% to 100%) and embedded in epoxy resin (Serva, Heidelberg, Germany) according to the TEM (Philips EM208S, Philips, Eindhoven, Netherlands) processing protocol described by Tay *et al*. (2003). After the beams were embedded in epoxy resin, undemineralized 90- to 100-nm-thick sections were prepared with an ultramicrotome (Ultracut S, Leica, Vienna, Austria) and examined without additional staining by TEM (Hashimoto *et al*., 2004).

Table 1. Mean Bond Strength Values (MPa) Based on Luting Resin and Surface Treatment Groups[a]

	CAPE	TiF_4	TiF_4 + CAPE	Control	Total
Bis-GMA[b]	20.8 ± 4.9^{A,1}	15.3 ± 4.8^{B,1,2}	11.3 ± 3.2^{C,2}	22.7 ± 6.3^{A,1}	17.5 ± 4.8^{1}
4-META/MMA[c]	19.7 ± 3.0^{A,1}	16.7 ± 4.7^{B,1}	8.9 ± 3.7^{C,2}	20.1 ± 5.9^{A,1}	16.3 ± 4.3^{1}
MMA[d]	13.9 ± 5.7^{B,2}	14.2 ± 4.3^{B,2}	6.3 ± 2.9^{C,3}	13.2 ± 5.2^{B,2}	11.9 ± 4.5^{2}
Total	18.1 ± 4.5^{A}	15.4 ± 4.6^{B}	8.8 ± 3.3^{C}	18.7 ± 5.8^{A}	

[a]Same uppercase letters represent no statistical difference between columns. Same numbers represent no statistical significant difference between rows (α = .05). CAPE, caffeic acid phenethyl ester; TiF_4, titanium tetrafluoride.
[b]Bisphenol glycydilmethacrylate (Variolink II, Ivoclar, Liechtenstein).
[c]4-methacryloyloxyethyl trimellitate anhydride methyl methacrylate (Super-Bond C&B, Sun Medical, Japan).
[d]Methyl methacrylate (Multilink Automix, Ivoclar).

One reading was carried out for each of the 6 specimens per group. Silver penetration into the hybrid layer was evaluated and graded in percentages for consecutively selected cross sections of the beams. Data were statistically analyzed by 1- and 2-way ANOVA tests with Bonferroni corrections (P = .05).

Scanning Electron Microscopy

Representative specimens from fractured surfaces for each cement group and subgroup were evaluated under scanning electron microscopy (JSM 5200, JEOL, Tokyo, Japan). Failure modes were classified as adhesive (> 75% between resin and dentin), cohesive (> 75% within resin and dentin), or mixed.

RESULTS

Microtensile Bond Strength Testing

In all test groups, only 3 pretest failures were recorded in the 4-META/MMA cement group (1 in control and 2 in TiF_4 subgroups).

Interaction terms were significant (P < .05). The differences in μTBS values of CAPE and control were not significant for Bis-GMA (20.8 ± 4.9, 22.7 ± 6.3, respectively) and 4-META/MMA (19.7 ± 3.0, 20.1 ± 5.9, respectively) (P > .05). TiF_4 (Bis-GMA, 15.3 ± 4.8; 4-META/MMA, 16.7 ± 4.7) and TiF_4 + CAPE (Bis-GMA, 11.3 ± 3.2; 4-META/MMA, 8.9 ± 3.7) groups resulted in significantly lower values than those of the control (P < .05) (Table 1). For MMA cement group, the differences among control (13.2 ± 5.2), TiF_4 (14.2 ± 4.3), and CAPE (13.9 ± 5.7) were not significant (P > .05), but the TiF_4 + CAPE (6.3 ± 2.9) group resulted in significantly lower values (P < .05). Regardless of the cement type, no significant difference was found between CAPE-applied groups (18.1 ± 4.5) and control (18.7 ± 5.8) (P > .05). TiF_4 group (15.4 ± 4.6) demonstrated significantly lower μTBS values, and TiF_4 + CAPE (8.8 ± 3.3) had the lowest values (P < .05). For all experimental chemical agent groups, Bis-GMA (17.5 ± 4.8) and 4-META/MMA (16.3 ± 4.3) exhibited similar μTBS values (P > .05) but MMA-based cement group (11.9 ± 4.5) showed significantly lower values (P < .05).

Failure Analysis

Failure modes were mainly mixed for etch and rinse–system resin groups: 82 of 168 and 77 of 168 for Bis-GMA (Fig. 1a-c) and 4-META/MMA cements (Fig. 1d-f), respectively. Predominantly adhesive failures at the interface without any dentin damage were observed in the self-etch resin group: 125 of 168 for MMA cement (Fig. 1g-i). For all cement types, control groups demonstrated mainly mixed type of failures (66 of 128); CAPE groups, mixed (59 of 126); TiF_4 groups, adhesive (70 of 128); and TiF_4 + CAPE, adhesive (98 of 128).

Nanoleakage Evaluation and Detection of Silver Nitrate at the Hybrid Layer

For each cement type, a trend for decreased nanoleakage was observed in the CAPE-applied groups, but the results were not significant per cement type when compared to the control group (P > .05) (Table 2).

TEM analyses revealed silver nitrate deposition in the hybrid layer of all untreated specimens (Fig. 2a-d). Among all CAPE-applied adhesive groups, almost no silver grains were detected for 4-META/MMA cement, and reduced amounts of silver penetration were observed for the other 2 composite resin-based cements (Figs. 2a,b). Silver penetration was observed at the adhesive layer of the composite resin cement groups. TiF_4 application at the adhesive-dentin interface did not prevent nanoleakage at the hybrid and/or adhesive layer (Fig. 2c). Combined application of TiF_4 + CAPE resulted in thicker adhesive layers (Fig. 2d). In MMA cement groups, silver staining was at both the whole hybrid layer and the adhesive layer.

DISCUSSION

In this study, the microtensile bond strength values of all TiF_4 + CAPE-applied cements to dentin decreased when compared with the control groups. Whereas CAPE application before adhesive application on dentin surface did not affect the bond strength of luting cements to dentin, TiF_4 decreased the bond strength of composite-resin cements to dentin except for MMA. Therefore, the null hypothesis was partially accepted.

Silver deposits were found predominantly on the adhesive side of the fractured beams and were distributed through the hybrid layer of specimens that were pretreated with CAPE and/or TiF_4 before luting with MMA cement. However, TEM images for CAPE- and/or TiF_4-applied etch-and-rinse 4-META/MMA cement revealed almost no silver deposits at the hybrid layer.

Dentin tissue itself is a source of resident proteinases, such as MMP-1, MMP-2, MMP-9, and MMP-20, and their release and activation can be induced by the low pH of etch-and-rinse systems (Chaussain-Miller *et al*., 2006). In spite of this, less or almost no activation of MMPs with self-etch adhesive systems was reported

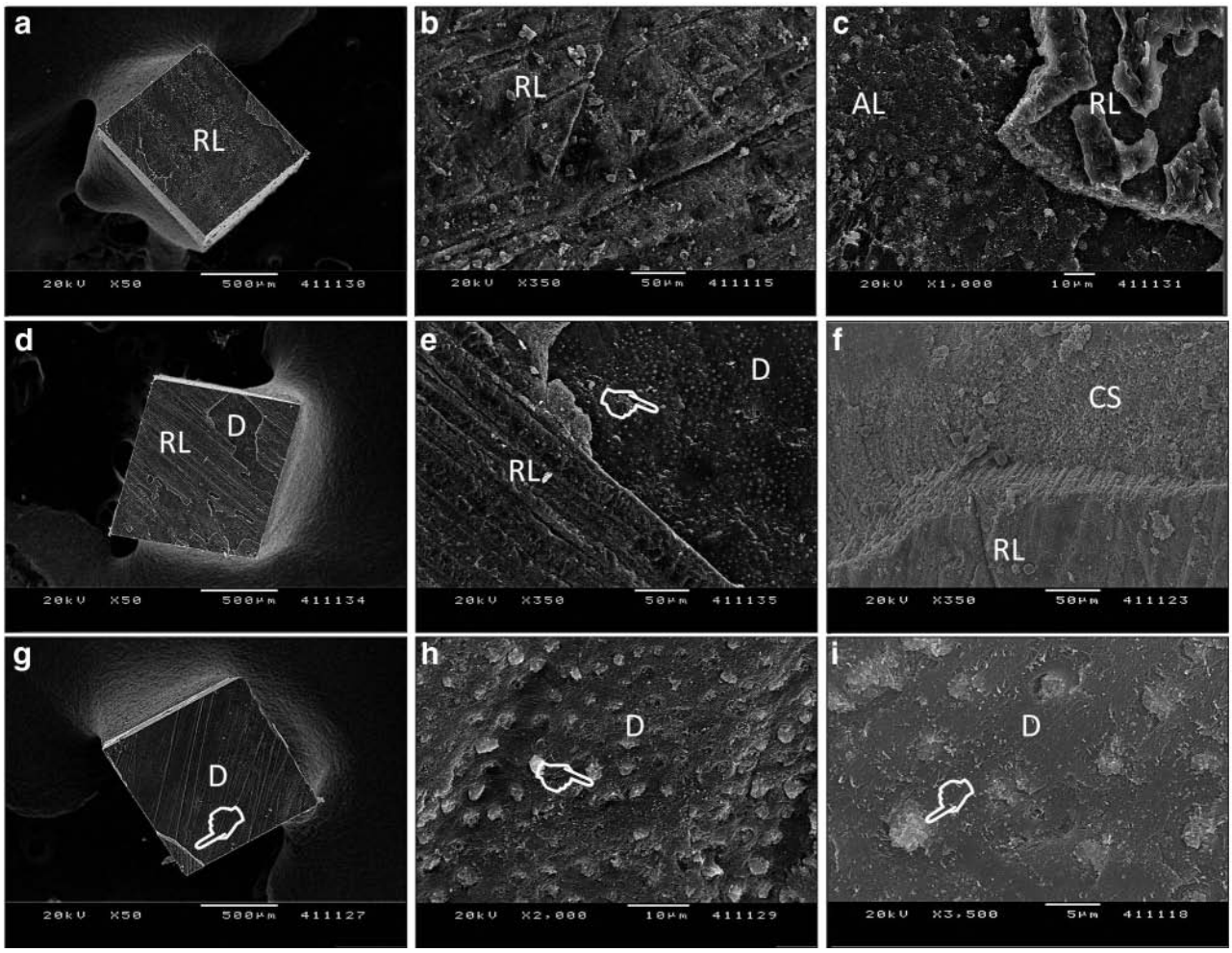

Figure 1. Representative images of the mixed failures from the Bisphenol glycydilmethacrylate (Variolink II, Ivoclar, Liechtenstein) cement group: **(a)** cohesive failure in the resin layer (RL; ×50), **(b)** RL at higher magnification (×350), and **(c)** typical mixed failure with partial cohesive failure of the RL and adhesive failure between the resin and the dentin (AL). Representative images of the mixed failures from the 4-methacryloyloxyethyl trimellitate anhydride methyl methacrylate (Super-Bond C&B, Sun Medical, Japan) cement group: **(d)** cohesive failure in the RL—RL and adhesive failure with dentin (D) exposure (×50), **(e)** RL delaminated from the dentin surface with notable tags (☞), and **(f)** one of the specimens showing delamination with a crack site (CS) within the dentin. Representative images of the adhesive failures from the methyl methacrylate (Multilink Automix, Ivoclar) cement group: **(g)** adhesive-type failure between the resin and the dentin (D)—note the small amount of RL left on the debonded surface (☞) (×50), **(h)** resin tags in the tubules (☞) with no RL on the failed surface, and **(i)** dentin tubules filled with resin at higher magnification (×3500).

Table 2. Mean Nanoleakage Amount (%) After the Application of Chemical Agents (n = 7) and Adhesives per Luting Resin Type[a]

	CAPE	TiF_4	TiF_4 + CAPE	Control	Total
Bis-GMA[b]	13 ± 2^{A,1}	24 ± 3^{B,1}	28 ± 3^{B,1}	30 ± 2^{B,1}	24 ± 7^{A}
4-META/MMA[c]	6 ± 1^{A,2}	12 ± 2^{B,2}	14 ± 1^{B,2}	18 ± 2^{B,2}	13 ± 4^{B}
MMA[d]	48 ± 5^{B,3}	60 ± 3^{C,3}	63 ± 4^{C,3}	59 ± 5^{C,3}	57 ± 7^{C}
Total	23 ± 18^{4}	32 ± 20^{4}	35 ± 21^{4}	35 ± 18^{4}	

[a]Same uppercase letters represent no statistical significant difference between columns. Same numbers represent no statistical significant difference between rows (α = .05). CAPE, caffeic acid phenethyl ester; TiF_4, titanium tetrafluoride.
[b]Bisphenol glycydilmethacrylate (Variolink II, Ivoclar, Liechtenstein).
[c]4-methacryloyloxyethyl trimellitate anhydride methyl methacrylate (Super-Bond C&B, Sun Medical, Japan).
[d]Methyl methacrylate (Multilink Automix, Ivoclar).

in a recent study (De Munck *et al.*, 2009). However, the obtained results after bond strength tests and TEM analyses in our work indicate that nanoleakage was more prominent at the hybrid layer and adhesive layer of the self-etching system used in our study. This was probably due to activated MMPs in the dentin. MMP activity at the coronal dentin based on immunohistochemistry, race, sex, tooth section orientation, tooth origin (maxilla or mandible), and tooth eruption status has been demonstrated to not correlate with the level of immunoreactivity (Boushell *et al.*, 2008). Yet in our study, nonerupted human third

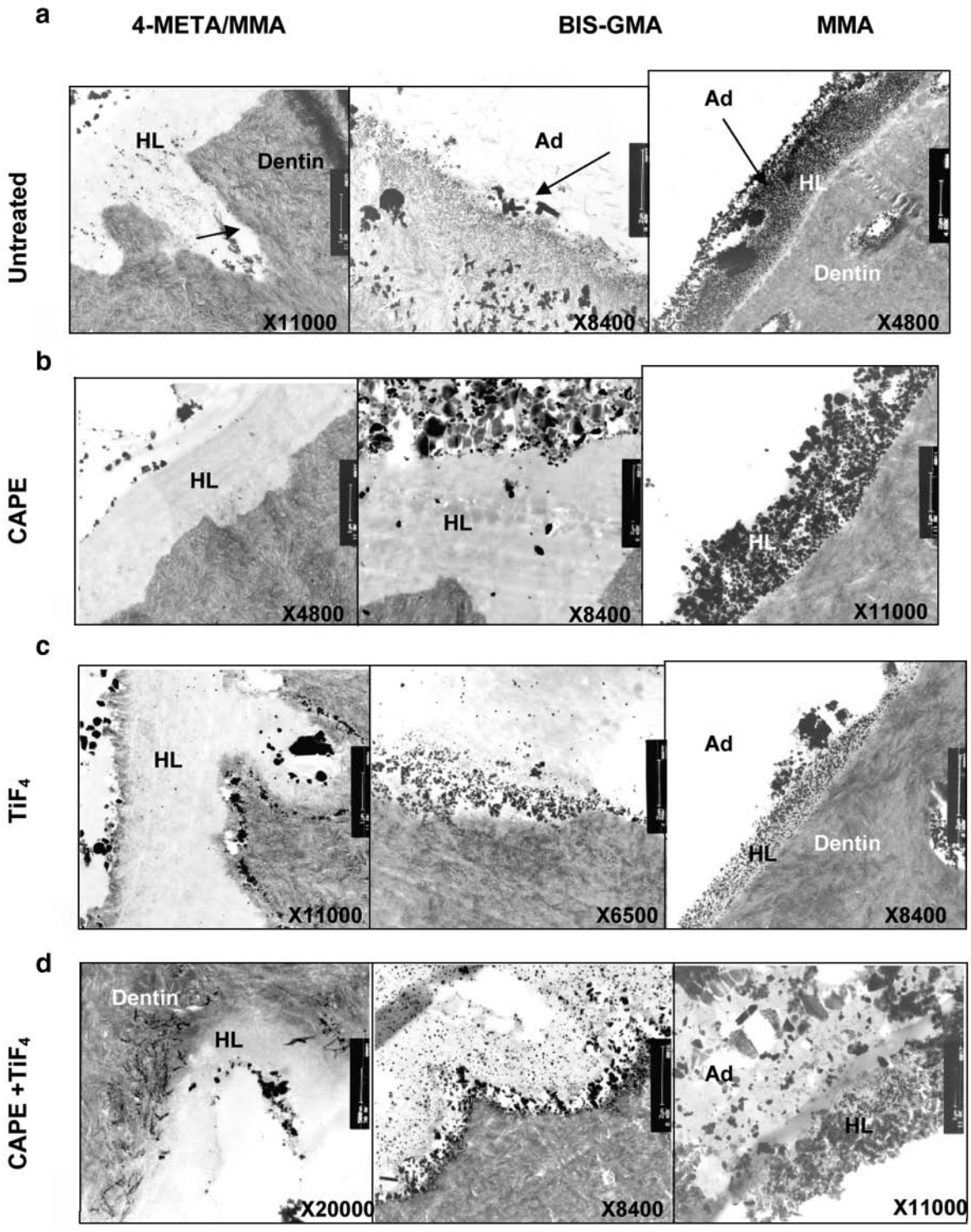

Figure 2. Silver nitrate staining of the hybrid layer (HL) and/or adhesive (Ad) in the *in vitro* thin-sliced (80-100 nm) human tooth model, with and without protective chemical agent treatment. Reticular type of silver penetration at the HL can be seen at the resin-dentin interface. In the methyl methacrylate (Multilink Automix, Ivoclar) cement group, silver staining is at the whole HL, as indicated by arrows. **(a)** In the 4-methacryloyloxyethyl trimellitate anhydride methyl methacrylate (Super-Bond C&B, Sun Medical, Japan) and Bisphenol glycydilmethacrylate (Variolink II, Ivoclar, Liechtenstein) cement groups applied with caffeic acid phenethyl ester (CAPE), almost no silver dye penetration was observed; however, extensive spotted type of nanoleakage pattern can be seen in the methyl methacrylate cement group. **(b)** titanium tetrafluoride (TiF_4) and TiF_4 + CAPE applications revealed spotted or granular nanoleakage patterns at the HL and Ad **(c, d)**.

molars were used to eliminate the possible aging affect from changing oral conditions. Low pH value and subsequent acid activation were related with collagenolytic and gelatinolytic activities (Chaussain-Miller *et al*., 2006). However, in a previous study, self-etching treatment on dentin surfaces (with milder acid application) did not have a significant effect on MMP-2 activity from the dentin tissue (Lehmann *et al*., 2009). The authors explained this finding by the fact that they had used adhesives as a clinical simulating procedure and obtained dentin slices, which is unlike previous study models where dentin powder mixed with self-etching adhesive was used and where liberation of gelatinolytic and collagenolytic activities was found to be more effective (Nishitani *et al*., 2006; Tay *et al*., 2006).

In this study, CAPE decreased nanoleakage at the hybrid layer, and it did not significantly decrease the bond strength of the cements used. TiF_4 and/or CAPE, however, could not prevent nanoleakage in the self-etched cement system used.

Chlorhexidine application on prepared dentin surface before bonding procedures has been shown to inhibit the MMP activity in dentin (Carrilho *et al*., 2007). However, bond strength of self-etch resin cements to dentin decreases after chlorhexidine application before adhesive applications, depending on the resin cement type (Hiraishi *et al*., 2009). Therefore, chlorhexidine application was not considered in this study.

Low pH (approximately 1.2) of TiF_4 favors the linking between titanium and oxygen of the phosphate group and leads to the formation of a titanium dioxide glaze-like layer on the surface (Alves *et al*., 2005). The reason for the decreased μTBS for TiF_4 and TiF_4 + CAPE groups in our study, especially for Bis-GMA and 4-META/MMA cements, might have resulted from this glaze-like layer formation on the prepared and treated dentin. However, TiF_4 treatment alone could not prevent nanoleakage at the hybrid layer with such a surface-layer formation. The optimization of TiF_4 treatment period of the dentin surfaces is a topic of an ongoing study.

Because CAPE had previously shown to inhibit MMP-9 enzyme activity in a dose-dependent manner (Jin *et al*., 2005) and dentin also contained MMP-9 enzyme, CAPE was used for the inhibition of nanoleakage and the inhibition of MMP activity in dentin for the first time for dental purposes in this study. Its optimal functioning under differing concentrations and its long-term durability effect under simulated clinical conditions are currently being investigated before proposing it as a new alternative agent for the inhibition of nanoleakage and hybrid-layer degradation.

When CAPE can be used in primers, in etchants, or as additive to adhesive comonomers, it may block the degradation cascade. The functioning mechanism of CAPE and other MMP-inhibiting chemical agents under occlusal forces by dynamic testing methods (Sano, 2006) should be evaluated to extrapolate clinically relevant results.

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